Establishment of genetic transformation system via *Agrobacterium* in tall fescue cultivar

QIAN Hai-feng [1*], Shaukat Ali [1, 2], HONG Liang[1], XU Hao [1]
[1] *College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou, 310014*
[2] *National Agricultural Research Centre, Park Road, Islamabad, Pakistan*

Abstract: Tall fescue (*Festuca arundinacea* Schreb.) is a cool-season turfgrass used on fairways in golf courses. The object of this study was to develop a more efficient, reliable, and repeatable approach in transforming the grass using *Agrobacterium (EHA105)*, where β-glucuronidase gene (*uidA*) was used as a reporter and hygromycin phosphotransferase gene (hyg) as a selectable marker. An effective expression of transgene was observed in transforming 2-month-old calli derived from mature seeds (cv. Bingo) cultured on MS medium supplemented with 9 mg·L^{-1} 2, 4-D. A two-step solid medium selection with increasing hygromycin concentration (from 30 to 50 mg·L^{-1}) was used to obtain resistant calli. Transgenic plants have been produced from many independent transformed calli. The presence of functional β-glucuronidase gene (*uidA*) was detected in hygromycin-resistant calli. Transgenic plants were regenerated and PCR and Southern blot confirmed transgene integration in the tall fescue genome.

Key words: Tall Fescue; *Agrobacterium tumefaciens*; Transformation; β-glucuronidase gene (*uidA*)
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Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is an open-pollinated, perennial, cool-season forage and turfgrass (Buckner *et al*. 1979). It was originally utilized as a forage grass in the United States, in recent years, as a turfgrass it has been gaining much popularity in China. Aside from contribution to soil development, stabilization, improvement and erosion control, tall fescue is also used to beautify the earth, enrich our lives, and provide recreation and enjoyment for the people. However, the growth of tall fescue is affected easily by various biotic stresses, the resistances of tall fescue to different stress factors need to be improved. A great amount of breeding effort has been conducted aiming at improving the turf quality of tall fescue, up to now, many new elite cultivars have been released (NTEP 1996).

As the most other crops, turfgrass improvement mainly relied on conventional breeding methods, in which the accessible genetic material is restricted by sexual reproduction, just like tall fescue, whose cultivars have been always bred by crossing several to more than ten parent lines (Bai *et al*. 2000). This situation raises the necessity of exploring other approaches for more efficient achievements. Modern plant biotechnologies based on our current understanding of biological systems at both the cellular and molecular levels, and have become increasingly associated with genetic engineering (Krans 1989).

To establish a genetic transformation system, it is necessary to have an explant competent for the transformation process, and an in vitro culture system, which permits a high frequency of regeneration. Furthermore, it is necessary to have a system of genetic transference that is simpler, more cheap, reproducible and independent of the genotype, which inserts the genetic sequence in a stable form (Birch 1997). Successful transformation for tall fescues has recently been reported, such as the use of polyethylene glycol- or electroporation-mediated DNA uptake into protoplasts derived from embryogenic cell suspensions of tall and red fescues (Ha *et al.* 1992; Wang *et al*. 1992; Spangenberg *et al.* 1994; Dalton *et al*. 1995; Kuai and Morris 1995; Bettany *et al*. 1998), microprojectile bombardment of tall and red fescue cell suspension cultures (Spangenberg *et al.* 1995; Cho *et al.,*2000), and silicon carbide fiber-mediated transformation of tall fescue cell suspension cultures (Dalton *et al*. 1998).

Monocotyledonous plants do not serve as a natural host for *Agrobacterium tumefaciens* (Potrykus 1990), however, within the last decade, a number of reports have appeared demonstrating successful *Agrobacterium*-mediated transformation of various monocot species, including rice (Hiei *et al.* 1994), maize (Ishida *et al.* 1996), wheat (Cheng *et al.* 1997), barley (Tingay *et al.* 1997; Trifonova *et al.* 2001), sugar cane (Arencibia *et al.* 1998), and sorghum (Zhao *et al*. 2000). Bettany *et al.* (2003) described, for the first time, *A. tumefaciens*-mediated transformation of the tall fescue. But previously, protoplasts or embryogenic cell suspension cultures were used as the only successful transformation targets for tall fescues. These approaches involved laborious steps and have been difficult to reproduce in terms of initiation and maintenance of cell suspension cultures, and also in isolation of protoplasts. The main objective of our work is to develop a genetic transformation method via *A. tumefaciens* for tall fescue, which does not utilize protoplasts or embryogenic suspension cultures, but used highly regenerative, green tissues from mature seed-derived embryogenic callus of tall fescues as transformation targets.

Materials and methods

Plant material

A commercial cultivar of tall fescue Cultivar 'Bingo', which is widely used for greens in golf courses in China and many other

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Biography: QIAN Hai-feng (1973-), male, Ph.D., associate professor in Zhejiang University of Technology. E-mail: hfqian@126.com
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*Corresponding author

temperate regions in the world, supplied by Shenghe Company China, was used in this study. Seeds were stored at 4℃ until used.

Bacterial strains and plasmids

Vector used to transform was based on a pCAMBIA1301 binary vector. The *A. tumefaciens* strain employed in the experiments was an agropine strain *EHA105*, and both of these were kindly donated by Genetic Institute of China. The T-DNA region of pCAMBIA1301 derivative is shown in Fig.1, which contains the hygromycin phosphotransferase (hyg)-coding sequence and *uidA* gene under control of the cauliflower mosaic virus (CaMV) 35S promoter.

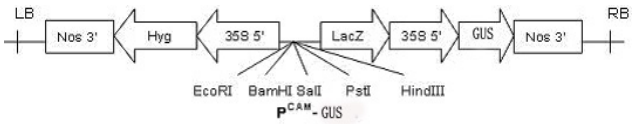

Fig. 1 Diagram of the plasmid construct used for grass transformation. The *uidA* gene and hygromycin resistance gene are under control of the cauliflower mosaic virus 35S promoter. LB: left border; RB: right border. Distances are not to scale.

Induction of embryogenic callus and *Agrobacterium*-mediated transformation

Cultivar 'Bingo' seeds were dehusked by stirring vigorously in 98% sulphuric acid for 90 s. The dehusked seeds were rinsed five times with sterilized water, followed by a rinse with alcohol (70%) for 3 min, and then surface sterilized in calomel (0.1%) for 20 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium MS9 containing MS basal salts and vitamins (Murashige and Skoog 1962), 30 g·L^{-1} sucrose, 500 mg·L^{-1} casein hydrolysate, 9 mg·L^{-1} 2,4-D, and 3 g·L^{-1} Phytagel. The pH of the medium was adjusted to 5.7 before autoclaving at 120℃ for 20 min. The culture plates containing prepared seed explants were kept in the dark at room temperature for 2 months. Embryogenic calli (formation of a very compact, white yellowish) were visually selected and cultured on MS9 medium for one week, and then transferred onto MS2 (free MS with 2 mg·L^{-1} 2,4-D) for one week in turn in the dark at room temperature. And the calli were transferred onto MBN medium (basal MS with 0.5 mg·L^{-1} BA and 0.5 mg·L^{-1} NAA) for three days. One day before agro-infection, the embryogenic calli were divided into 1- to 2-mm pieces and placed on MBN medium containing 100 μM acetosyringone. A 10 mL aliquot of *Agrobacterium* suspension (OD=0.6 at 660 nm) was then applied to each piece of callus, followed by three days of co-cultivation in the dark at 25°C. Infected calli were rinsed two times with sterilized water, and then immersed into MS liquid medium with 500 mg·L^{-1} carbenicillin 15 min to rinse *Agrobacterium* drastically. For the antibiotic treatment steps, the callus was then transferred and cultured for one week on MS2 medium plus 500 mg·L^{-1} carbenicillin to suppress bacterial growth. Subsequently, for selection, the callus was moved to callus-induction medium containing 500 mg·L^{-1} carbenicillin and 30 mg·L^{-1} hygromycin for one week and then transferred onto 50 mg·L^{-1} hygromycin for selection to eliminate pseudo-resistant calli (Qian *et al*. 2004). Antibiotic treatments and the entire selection process were performed at room temperature in the dark. During antibiotic selection, bright yellow hygromycin resistant calli started appearing. For plant regeneration, the resistant calli were moved to regeneration medium (MS basal medium, 30 g·L^{-1} sucrose, 100 mg·L^{-1} myo-inositol, 2 mg·L^{-1} BAP, 0.5 mg·L^{-1} NAA, 50 mg·L^{-1} hygromycin, and 2 g·L^{-1} Phytagel), and then incubated in a 25°C growth chamber under a cycle of 16-h illumination (100 μmol·m^{-2}·s^{-1}) and 8-h darkness. In approximately three weeks, shoots of plantlets were recovered and transferred into new regeneration medium. Independent transgenic plantlets at the height of 7-10 cm with vigorous root development were transferred to potting soil in the greenhouse.

***Agrobacterium* protocol**

The *A. tumefaciens* strain *EHA105* containing pCAMBIA1301 was streaked out on YEP medium (10 g·L^{-1} peptone, 10 g·L^{-1} yeast extract, 5 g·L^{-1} NaCl, and agarose 15 g·L^{-1}) supplemented with 50 mg·L^{-1} kanamycin and grown at 28°C for colonies to appear. A single colony was transferred to 3 mL YEP liquid medium containing the same selective antibiotics and the culture was shaken overnight at 28°C at 260 rpm. The grown bacteria were sampled 100 μL into 25 mL AB medium (5 g·L^{-1} glucose; 1 g·L^{-1} NH_4Cl; 0.3 g·L^{-1} $MgSO_4·7H_2O$; 0.15 g·L^{-1} KCl; 10 mg·L^{-1} $CaCl_2$; 2.5 mg·L^{-1} $FeSO_4·7H_2O$; 3 g·L^{-1} K_2HPO_4; and 1.15 g·L^{-1} $NaH_2PO_4·H_2O$) (Chilton 1974) and grown overnight to obtain an A600 of 0.5-0.6 (A600 1.0 corresponds to 1×10^8) on the same condition (28°C, 260 rpm), and then also sampled 100 μL bacteria into 25 mL AB medium supplemented with 100 μM acetosyringone (AS; Fluka Chemika, Switzerland) cultured overnight on the same condition. The bacterial cells were centrifuged at 5 000 rpm in eppendorf for 5 min and the pellet was dissolved in MS medium supplemented with 100 μM AS and 5% glucose. The suspension was then diluted such that the final OD600 reading was about 0.6 for co-transformation.

Histochemical uidA assay

Histochemical assay of *uidA* gene expression was conducted according to Jefferson (1987). After co-cultivation, calli were washed with sterile water and covered with filter-sterilized *uidA* substrate buffer (Klein *et al*. 1988) containing 20% methanol (v:v) and incubated at 37°C for 16–24 h. The *uidA* substrate buffer consisted of 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.3% (w:v) 5-bromo-4-chloro-3-indolyl-β-D-glucuronide acid, 0.1 M sodium phosphate (pH 7.0), and 0.3% (v:v) Triton X-100.

Polymerase chain reaction and southern analysis

PCR analysis

Transgenic plant verification was performed with putative transformants and wild-type tall fescue plants by PCR and Southern blot analyses. Total genomic DNA was extracted from green leaves of putative transformants and wild-type plants using the CTAB method (Wilkie 1997). PCR analysis was carried out using the following primers, *uidA* primers: 5'- GGGATCCATCGCAGCGTAATG-3', 5'-GCCGACAGCAGCAGTTTCATC-3'; hyg primers: 5'-TAGGAGGGCGTGGATATGGC-3', 5'-TACACAGCCATCGGTCCAGA-3'). The PCR conditions were as follows: 94℃ for 5 min; 94°C for 1 min, 61°C (Hyg also for 61°C) for 1 min, 72°C for 1 min comprising 30 cycles, followed by the final cycle carried out at 72°C for 5 min. The amplified products were electrophoresed on 1% agarose gel staining with ethidium bromide. The expected amplified products for these reactions were 563 bp, 852 bp fragments for *uidA* and hyg genes, respectively.

Southern analysis

Ten μg DNA was digested with HindIII, electrophoresed in a 0.8% agarose gel, and subsequently transferred to a Hybond N+ nylon membrane. The 852 bp fragment containing the hyg gene coding region was used as a probe. Probe labeling, membrane pre-hybridization and hybridization were carried out according to the hybridization-kit instructions supplied by Amersham Pharmacia Biotech Company (http://www4.amershambiosciences.com). After hybridization, the membrane was sealed with a plastic sheet and exposed to X-ray film.

Results

Efficient delivery of transgenes to tall fescue through *Agrobacterium*-mediated transformation

Before transformation, callus induction and regeneration system of tall fescue have been established, and the ratios are 66.7% and 73.3%, respectively (Qian *et al*. 2002). To set up an effective regeneration system suitable for *Agrobacterium*-mediated transformation, we also needed to determine the minimal concentrations of hygromycin and carbenicillin in the selection medium. Within 1 month of culture, the minimal concentration of hygromycin (50 mg·L^{-1}) via untransformed control calli was estimated by counting the dead calli on selection medium containing various concentrations of hygromycin. Carbenicillin inhibits the growth of *A. tumefaciens* during transformation on selection medium, addition of carbenicillin showed no toxicity to calli even at a concentration of 500 mg·L^{-1}.

One of the most critical factors to insure success in this transformation protocol is the efficient formation and maintenance of embryogenic callus from seeds. This is absolutely essential for efficient transformation and plant regeneration. Using the media and experimental conditions described (Materials and methods), we can see that calli induced from MS9 medium are non-embryonic calli which were unorganized and had loosely tentive, washy and white appearance, while subcultured according to our methods, high-quality friable embryogenic callus can be obtained from the seeds of tall fescue cv. Bingo (Fig. 2), and this kind of calli are easy to be infected by *Agrobacterium*.

We herein used a reporter *uidA* gene construct (Fig. 1) with a hygromycin resistance gene as the selectable marker to evaluate the potential effect of *Agrobacterium* infection on callus growth and development as well as to assess the effectiveness of *Agrobacterium*-mediated transformation in grass. Transient *uidA* gene expression was used to monitor T-DNA delivery into turfgrass embryogenic callus following *Agrobacterium* infection and co-cultivation. An average of approximately 30% of embryogenic callus expressed *uidA*A after co-cultivation (Data not shown). The transformed calli were then cultured on a hygromycin-containing medium, and resistant calli were obtained six weeks after co-cultivation. Nearly all the calli still showed strong *uidA* expression (Fig. 3). The majority of plants regenerated from these calli on hygromycin-containing medium were observed to grow normally (Fig. 3).

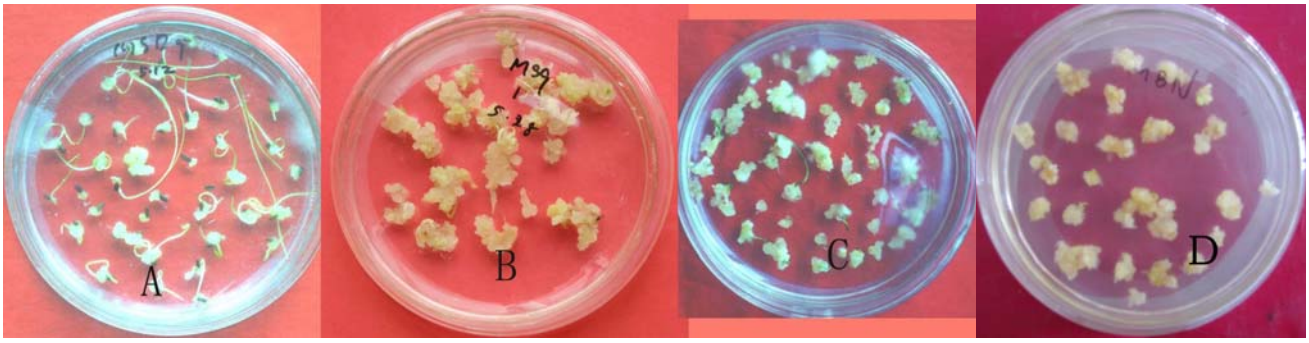

Fig. 2 Embryogenic callus induction and subculture.
A: Embryogenic callus initiated from seeds. B: Callus cultured on MS9. C: Callus subcultured on MS2. D: Callus subcultured on MBN.

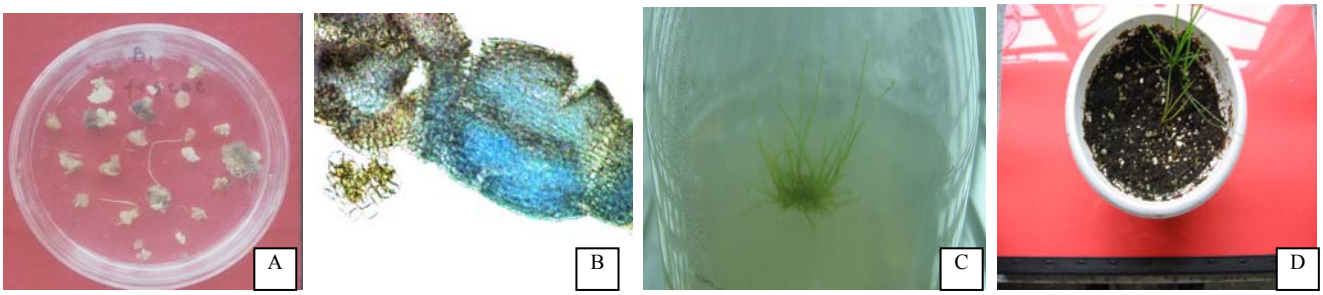

Fig. 3 Transgene expression in the transformed embryogenic callus and transgenic line. A: White-yellow resistant calli formed from brown calli which infected by Agrobacterium on selective medium. B: Cell structure of histochemical staining of GUS activity in transformed callus (by mitic digital microscope, micro-optic industrial Group Co., Ltd). C: Transgenic plant grown on regeneration medium. D: Transgenic plant grown in pot

Molecular analyses of putative transformants

Leaf tissues from independent Hyg-resistant and *uidA* positive lines, the regrowth shoots of tall fescue on soil pot, were used for PCR and/or Southern analyses. Four *uidA*-positive lines were also found to be PCR-positive using *uidA* and hyg primers. The expected fragments of 563 bp (*uidA*) and 852 bp (hyg) were observed in the putative transformants and were absent in non-transformed plants (Fig. 4).

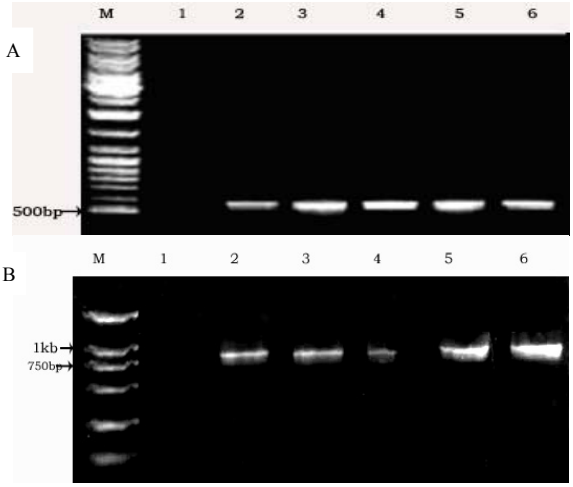

Fig. 4 Ethidium bromide-stained agarose gel showing PCR products (A: *uidA* 563bp, B: Hyg 852bp fragment) amplified from the genomic DNA. M: DNA molecular-weight ladder; 1: the template is the control as negative control; 2-5: putative transgenic plantlets; 6: plasmid as positive control.

Southern blot analysis was performed on four *uidA* positive and PCR-positive clones of tall fescue. The T-DNA region of the binary vector has a single internal HindIII cleavage site. These four *uidA*-positive and PCR-positive clones had over 3 hybridizing bands (Fig. 5), therein the second line had three hyg copies, the third and fourth had over ten copies. Considering the hybridizing bands of three and four are the same size, we speculated that these two transgenic lines may be not the independent clones, and the fifth line had no band for the genomic DNA that we extracted was degraded. As expected, no bands were detected in the genomic DNA from the non-transformed control (Fig. 5).

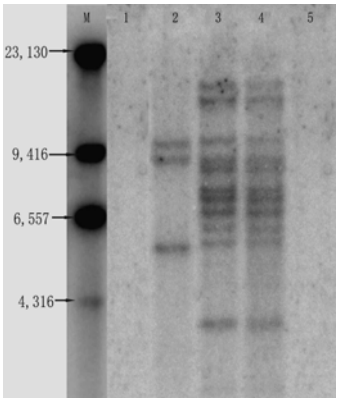

Fig. 5 Southern analysis for the *uidA* reporter gene in four randomly selected independent transgenic clones of cv. Bingo. DNA samples were digested with HindIII. Lanes: M: DNA ladder; 1: non-transformed control, 2-5: transgenic clones of cv. Bingo.

Discussion

Endophytes are organisms that are contained or grow entirely within plants including seeds and spend all or nearly all of their life cycles in their hosts (Siegel 1987; Breen 1993a, b). *Acremonium coenophialum* is one kind of endophytic fungi, how to control these endophytic fungi in tissue culture is difficult, and it is also the first step in achieving in vitro regeneration of tall fescue. Several basic techniques were used to sterilize leaf explant which consisted of surface sterilization in 70% ethanol for 5 s, followed by 5 min in a 0.525% NaClO solution (w/v) (i.e., 10% commercial bleach solution, v/v), and three sterile water washes (Latch and Christensen 1985). Tuite (1969) used 0.525% NaClO solution for 2 min followed by flaming explants after dipping in 70% ethanol (v/v), but contamination rate is over 60%. Increasing the exposure time to NaClO was adjusted until a low contamination rate was found, along with low explant death caused by overexposure to NaClO (Kearney *et al.* 1991). However, the techniques above were not fitted in our experiment when we used mature seeds as explant. We had dehulled the seeds manually and surface-sterilized in 70% ethanol for 3 min, followed by immersion in a solution of 0.1% $HgCl_2$ for 15 min, and 4 consecutive 1 min washing with sterile water (Qian *et al.* 2002), but it was time-cost way. We herein used 98% sulphuric acid to dehusk for 90 s (If stirred more than 2 min, seeds will lose activity), and then followed as Qian (2002), the ratio of contamination was lower than 5%.

In addition to the problems associated with establishing sterile technique, tall fescue was found to be largely recalcitrant to unresponsive to many plant growth regulators (Bai 2001). Until recently, suitable concentrations for auxins and cytokinins have been reported for in vitro culture of tall fescue explants (Bai *et al.* 2000; Bai 2001). The level of BAP (a kind of cytokinins) was beneficial of callus induction (Bai *et al* 2000), but the opposite results were obtained in our experiments (Data showed in Qian *et al.* 2000) because of using different cultivar maybe. Also, calli were not produced when seeds were cultured on MS medium supplemented with TDZ (Data not shown). So, it was thought that the most important recalcitrant factor in tall fescue tissue culture is only the concentration of 2, 4-D.

The method of plant transformation was improved on rice transformation experiments of our research group (Qian *et al.* 2004). Molecular experiment results confirmed the stable integration transgenic tall fescue via *A. tumefaciens*-mediated transformation. In conclusion, we have developed a protocol for efficient adventitious shoot regeneration, transient transformation and production of transgenic plants of different tall fescue cultivars. The system uses suitable regeneration conditions based on the response of stock cultures, co-cultivation of calli with *A. tumefaciens* for three days in the presence of 100 μM AS, removal of excess *A. tumefaciens* by soaking twice in water and liquid MS medium containing 500 $mg \cdot L^{-1}$ carbenicillin prior to selection, selection and regeneration of transformed cells on MS containing 50 $mg \cdot L^{-1}$ hygromycin and 500 $mg \cdot L^{-1}$ carbenicillin. Following this protocol, stable transgenic plants of tall fescue were obtained. Hyg-resistant/ *uidA*-positive clones were found to be PCR-positive, and PCR-positive shoots tested were subsequently confirmed by Southern analysis to be stably transformed and the transformation method devised herein would appear to be applicable to other cultivars tall fescue and turfgrasses.

References

Arencibia, A.D., Carmona, E.R., Tellez, P., *et al*. 1998. An efficient protocol for sugarcane (*Saccharum* spp. L.) transformation mediated by *Agrobacterium tumefaciens* [J]. Transgenic Research, **7**: 213–222.

Bai, Y., Qu, R.A. 2000. Evaluation of callus induction and plant regeneration in twenty-five turf-type tall fescuecultivars [J]. Grass and forage science, **55**: 326–330.

Bai, Y. 2001. Tissue culture and genetic transformation of tall fescue (Ph.D. thesis) [D]. Bell and Howell Information and Learning 300 North Zeeb Road, Ann Arbor, MI 48106–1346 USA.

Bettany, A.J.E., Dalton, S.J., Timms, E., *et al*. 2003. *Agrobacterium tumefaciens*-mediated transformation of *Festuca arundinacea* (Schreb.) and *Lolium multiflorum* (Lam.) [J]. Plant Cell Report, **21**: 437–444.

Bettany, A.J.E., Dalton, S.J., Timms, E., *et al*. 1998. Stability of transgene expression during vegetative propagation of protoplast derived tall fescue (*Festuca arundinacea* Schreb.) plants [J]. Journal Experimental Botany, **49**: 1797–1804.

Birch, R.G. 1997. Plant transformation: problems and strategies for practical application [J]. Annual Review of Plant Physiology and Plant Molecular Biology, **48**: 297–326.

Breen, J.P. 1993a. Enhanced resistance to fall armyworm in *Acremonium endophyte*-infected turfgrasses [J]. Journal of Economic Entomology, **86**: 621–629.

Breen, J.P. 1993b. Enhanced resistance to three species of aphids (Homoptera: Aphididae) in *Acremonium endophyte*-infected turfgrasses [J]. Journal of Economic Entomology, 86:1279–1285

Buckner, R.C., Powell, J.B., Frakes, R.V. 1979. Historical development. In: Buckner, R.C. and Bush, L.P. (eds.) Tall Fescue [M]. Madison, WI, USA: American Society of Agronomy, pp1–8.

Cheng, M., Fry, J.E., Pang, S., *et al*. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens* [J]. Plant Physiology, **115**: 971–980.

Chilton, M.D., Curier, T.C., Farrand, S.K., *et al*. 1974. *Agrobacterium trmefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors [J]. Proceedings of the National Academy of Sciences USA, **71**: 3672–3676.

Cho, M.J., Ha, C.D., Lemaux, P.G. 2000. Production of transgenic tall fescue and red fescue plants by particle bombardment of mature seed-derived highly regenerative tissues [J]. Plant Cell Report, **19**: 1084–1089.

Dalton, S.J., Bettany, A.J.E., Timms, E., *et al*. 1995. The effect of selection pressure on transformation frequency and copy number in transgenic plants of tall fescue (*Festuca arundinacea* Schreb.) [J]. Plant Science, **108**: 63–70.

Dalton, S.J., Bettany, A.J.E., Timms, E., *et al*. 1998. Transgenic plants of *Lolium multiflorum, Lolium perenne, Festuca arundinacea* and *Agrostis stolonifera* by silicon carbide fibremediated transformation of cell suspension cultures [J]. Plant Science, **132**: 31–43.

Ha, S.B., Wu, F.S., Thorne, T.K. 1992. Transgenic turf-type tall fescue *Festuca-arundinacea* Schreb. Plants regenerated from protoplasts [J]. Plant Cell Report, **11**: 601–604.

Hiei, Y., Ohta, S., Komari, T., *et al*. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA [J]. Plant Journal, **6**: 271–282.

Ishida, Y., Saito, H., Ohta, S., *et al*. 1996. High efficiency transformation of maize (*Zea mays*) mediated by *Agrobacterium tumefaciens* [J]. Nature Biotechnology, **4**: 745–750.

Jefferson, R.A. 1987. Assaying chimerical genes in plant: The GUS gene fusion system [J]. Plant Molecular Biology Report, **5**: 387–405.

Kearney, J.F., Parrott, W.A., Hill, N.S. 1991. Infection of somatic embryos of tall fescue with *Acremonium coenophialum* [J]. Crop Science, **31**: 979–984.

Klein, T.M., Gradziel, T., Fromm, M.E., *et al*. 1988. Factors influencing gene delivery into Zea mays cells by high velocity microprojectiles [J]. Bio/Technology, **6**: 559–563.

Krans, J.V. 1989. Biotechnology advances in turfgrass. In: Takatoh, H. (ed.) Proc. Int. Turfgrass Res. Conf., 6th (Tokyo)[M]. Int. Turfgrass Soc. and Jpns. Soc. Turfgrass Science. pp11–15.

Kuai, B., Morris, P. 1995. The physiological state of suspension cultured cells affects the expression of the glucuronidase gene following transformation of tall fescue (*Festuca arundinacea*) protoplasts [J]. Plant Science, **110**: 235–247.

Latch, G.C..M., Christensen, M.J. 1985. Artificial infections of grasses with endophytes [J]. Annual Applied Biology, **107**: 17–24.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures [J]. Physiology Plant, **15**: 473–497.

National Turfgrass Evaluation Program (NTEP). 1997. National Tall Fescue Test-1992. Final Report 1993–1995. NTEP No. 96–13. Beltsville, MD: USDA-ARS.

Potrykus, I. 1990. Gene transfer to cereals: an assessment [J]. Bio/Technology, **8**: 535–542

Qian Haifeng, Xue Qingzhong. 2002. The effect of hormones on callus induction and plantlet regeneration of tall fescue (*Festuca arundinacea*) [J]. Grassland of China, **1**: 46–50. (in Chinese)

Qian, H.F., Zhang, X.Y., Xue, Q.Z. 2004. Factors affecting the callus induction and GUS transient expression in indica rice Pei'ai64s [J]. Pakistan Journal Biology Science, 7: 615–619.

Siegel, M.R. 1987. Fungal endophytes of grasses [J]. Annual Review Phytopathology, **25**: 293–315.

Spangenberg, G., Wang, Z.Y., Nagel, J., *et al*. 1994. Protoplast culture and generation of transgenic plants in red fescue (*Festuca rubra* L.) [J]. Plant Science, **97**: 83–94.

Spangenberg, G., Wang, Z.Y., Wu, X.L., *et al*. 1995. Transgenic tall fescue (*Festuca arundinacea*) and red fescue *(F. rubra*) plants from microprojectile bombardment of embryogenicvsuspension cells [J]. J. Plant Physiology, **145**: 693–701.

Tingay, S., McElroy, D., Kalla, R., *et al*. 1997. *Agrobacteriumtumefaciens*-mediated barley transformation [J]. Plant Journal, **11**: 1369–1376.

Trifonova, A., Madsen, S., Olesen, A. 2001. *Agrobacterium*-mediated transgene delivery and integration into barley under a range of in vitro culture conditions [J]. Plant Science, **162**: 871–880.

Tuite, J. 1969. Plant pathological methods [M]. Burgess Publ. Co., Minneapolis.

Wang, Z.Y., Takamizo, T., Iglesi, V.A., *et al*. Transgenic plants of tall fescue (*Festuca arundinacea* Schreb.) obtained by direct gene transfer to protoplasts [J]. Bio/Technology, **10**: 691–696.

Wilkie, S. 1997. Isolation of total genomic DNA. In: Clark, M.S. (Ed.), Plant Molecular Biology—A Laboratory annual [M]. Springer-Verlag Berlin: p3–15

Zhao, Z.Y., Cai, T., Tagliani, L., *et al*. 2000. *Agrobacterium*-mediated sorghum transformation [J]. Plant Molecular Biology, **44**: 789–798.